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Review article

Xenografted tissue models for the study of human endometrial biology

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ABSTRACT

The human endometrium undergoes extensive morphological, biochemical and molecular changes under the influence of female sex steroid hormones. Besides the fact that estrogen stimulates endometrial cell proliferation and progesterone inhibits this proliferation and induces differentiation, there is limited knowledge about precise molecular mechanisms underlying human endometrial biology. The importance of paracrine signaling in endometrial physiology explains why *in vitro* culture of endometrial cells has been challenging. Researchers, therefore, have developed alternative experimental *in vivo* models for the study of endometrial biology. The objective of this review is to summarize the recent developments and work on these *in vivo* endometrial research models. The *in vivo* recombinant tissue models in which wild-type endometrial cells are combined with endometrial cells from a gene-targeted mouse strain followed by xenografting to host mice have been critical in confirming the significance of paracrine signaling between the epithelium and stroma in the growth regulation of the endometrium. Additionally, these studies have uncovered differences between the mouse and human, emphasizing the need for the development of experimental models specifically of the human endometrium. Recently, xenotransplants of human endometrial fragments into the subcutaneous space of host mice and endometrial xenografts of dissociated and recombined epithelial and stromal cells beneath the kidney capsule of immunodeficient host mice have proven to be highly promising tools for *in vivo* research of endometrial functions. For the first time, the latter approach provides an immense opportunity for the application of genome engineering, such as targeted ablation of endometrial genes for example by using CRISPR/CAS9 system. This research will begin to elucidate the functional role of specific genes in this complex tissue. Another advantage of xenotransplantation and xenograft models of the human endometrium is their use to investigate endometrial effects of new compounds and drugs without needing to give them to women. Underpinning the molecular mechanisms underlying endometrial functions is critical to ultimately advance our understanding of endometrial pathophysiology and develop targeted therapies to prevent and cure endometrial pathologies as well as enhance endometrial function when it is desired for fertility.

1. Introduction

The human endometrium is a complex dynamic tissue comprised of several cellular compartments including luminal and glandular epithelium, stromal fibroblasts, immune cells and endothelial cells. A muscular layer known as the myometrium surrounds the endometrium. The endometrium is a classical sex steroid hormone target tissue and exhibits unique properties of extensive cyclic regeneration and transformation under the control of 17ß-estradiol (E2) and progesterone (P) in the preparation for embryo implantation. Both E2 and P elicit their effects through the cognate receptors that are members of the steroid receptor superfamily, ESR and PGR respectively (Evans, 1988; Hamilton et al., 2017). Estrogen receptor alpha (ESR1) appears to be the predominant receptor in mediating E2 signaling in the uterus (Couse et al., 1995). If no pregnancy is established, prior exposure of the endometrium to P is necessary to induce monthly menstrual bleeding that in turn is caused by the loss of P stimulation to allow a new cycle to start. Inadequate exposure or reduced responsiveness of the endometrium to P additionally exacerbates the development of endometrial pathology, including endometrial hyperplasia and adenocarcinoma as well as endometriosis that are driven by E2 (Burney et al., 2007). Thus, the physiological responses of the human endometrium to

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the sex steroid hormones translate to its most important functions: continuing reproduction of species and prevention of pathology. The underlying molecular mechanisms of both functions still remain largely unknown.

The cyclic hormonal changes of the human endometrium were described in great detail by Noyes and Haman at the morphological and histological level using human endometrial biopsies collected at different time points of the menstrual cycle (Noyes and Haman, 1953). Their systematic analysis of endometrial biopsies across the menstrual cycle demonstrated cell proliferation (E2 effect) followed by a sequence of distinct secretory changes after ovulation (predominant P effect), finally culminating in tissue breakdown and menstrual shedding of the endometrium (withdrawal of P). These original studies have been refined over the years using immunohistochemical (IHC) markers of cell proliferation, coupled with expression of cell cycle regulated proteins and steroid receptors that together with quantitative approaches in staged endometrial biopsies confirmed the Noyes staging and form the basis of current understanding (Niklaus et al., 2007). Similarly, global gene expression studies of the whole human endometrium defined proliferative, early secretory, mid-secretory and late secretory endometrium into specific categories based on transcriptional and microRNA profiles, indicating the significance of the coordinated sequence of molecular events in response to sex steroids (Talbi et al., 2006; Borthwick et al., 2003; Kuokkanen et al., 2010; Diaz-Gimeno et al., 2014).

For the past decades Murid species particularly rats and mice have been used as research models to expand our knowledge on the regulation of uterine epithelial cell proliferation and differentiation. Although these two species do not have menstrual cycles, they show very similar patterns of responsiveness to E2 and P during their estrous cycle and subsequent preparation for implantation following copulation as observed in humans during the menstrual cycle. Furthermore these states can be completely replicated in hormone treated ovariectomized adult females (Tong et al., 2008). Using these species, early observations resulted in key findings that pointed to a paradigm of paracrine signaling in the growth control of the mouse uterine epithelium. When E2 was administered to neonatal mice, it induced DNA synthesis in uterine epithelial cells even though E2 binding activity was not present in these cells that lack ESR1 at this early developmental stage (Bigsby and Cunha, 1986; Bigsby et al., 1990). However, unlike the epithelium, the mesenchymal cells of the early developmental uteri express ESR1, prompting a hypothesis that signaling from the mesechymal cells would indirectly mediate the epithelial response to E2. Support for a stromally synthesized factor on the epithelial proliferative response to E2 came from *in vitro* experiments where E2 was ineffective in inducing mitogenic activity in pure epithelial cell culture (Tomooka et al., 1986), but showed proliferation when recombined with stromal cells and grown in xenotransplants (Cooke et al., 1986). Furthermore, conditional genetic ablation of *Esr1* and *Pgr* in the luminal and glandular epithelium has indicated the requirement for *Esr1* in the stroma for the E2 induction of DNA synthesis although inhibition by P required PGR expression in these epithelial cells (Winuthayanon et al., 2010; Franco et al., 2012). These conditional ablation experiments along with others have further emphasized reciprocal paracrine signaling between the stroma and epithelial compartments in the control of uterine preparation for implantation (Adams and DeMayo, 2015).

Mechanistic studies of endometrial functions in human have been hindered by the lack of experimental models suitable for reproducing dynamic changes of the menstrual cycle in this remarkably regenerative tissue. Observation of the transformation of the human endometrium during a single menstrual cycle will take approximately 4 weeks and involve multiple sampling of many women to produce statistically valid data. As the long-term *in vitro* culture of endometrial epithelial cells in combination with stromal cells is difficult, there appears to be a limited possibility to maintain truly hormonally receptive epithelial cells in culture for such long duration. Nevertheless, there have been recent advances enabling the culture of endometrial epithelial organoid cultures in matrigel that respond to E2 and P with appropriate changes in gene expression patterns (Turco et al., 2017). However even in these organoid cultures, the epithelial cells were not shown to proliferate in response to E2 perhaps because they lack stromal elements. Thus in an effort to advance our understanding of the mechanisms of steroid hormone actions in human endometrial functions, a variety of experimental models *in vivo* have been developed. In this review, we will discuss the *in vivo* models that involve the xenotransplantion into immunodeficient host mice of functional human endometrium using either endometrial tissue fragments or dispersed endometrial epithelial and stromal cells that are recombined before implantation. Additionally, we will discuss the application by our laboratory of the human endometrial recombined xenograft model for mechanistic studies to unravel cyclic endometrial changes under the influence of E2 and P control.

2. Epithelial and stromal cell tissue recombinants of endometrium

Genetically engineered mice, in particular *Esr1* and *Pgr* null mutant mice, were developed to specifically interrogate the sex steroid actions in uterine epithelial cell growth (Hamilton et al., 2017; Adams and DeMayo, 2015). The results from a series of studies using heterotypic endometrial tissue recombinants of *Esr1* and *Pgr* knockout mouse models transplanted into mice have shed light on paracrine interactions regulating mitogenesis of the endometrial epithelium. For example, analysis of tissue recombinants prepared using the uteri of wild-type and *Esr1* knockout (ERKO) mice demonstrated that E2-induced epithelial mitogenesis and E2-induced downregulation of PR in the epithelium is mediated by stromal ESR1 without epithelial ESR1 contribution to this process (Kurita et al., 2000; Cooke et al., 1997). In a further study, Kurita et al. investigated epithelial and stromal PR participation in the inhibition of epithelial DNA synthesis using tissue recombinants of uteri from wild-type and progesterone receptor knockout (PRKO) mice (Kurita et al., 1998). The results indicated that stromal PR mediates the P inhibitory effect on E2-induced uterine epithelial DNA synthesis, whereas epithelial PGR was not sufficient for this inhibition (Kurita et al., 1998). However, these data from recombinant tissues are in contradistinction to the targeted ablation of *Pgr* in the mouse uterine luminal epithelium that showed the requirement for epithelial PGR to inhibit E2-induced DNA synthesis (Franco et al., 2012). Thus, although the tissue recombinant studies point to the significant role of epithelial-stromal paracrine actions in P control of epithelial cell growth, genetic ablation in a cell type specific fashion provided contradictory results. Thus care does need to be taken in their interpretation as there maybe compensatory mechanism in play as the recombined tissue is in a foreign environment, the recombinants do not contain all uterine cell types and the timing of P action is different (Wetendorf and DeMayo, 2014; Vasquez and Demayo, 2013).

More recently, the role of specific paracrine factors, such as IGF1 and FGFs, has been identified to mediate hormonal effects between epithelial and stromal compartments in the mouse uterus (Zhu and Pollard, 2007; Li et al., 2011). Pierro et al. described a co-culture system of human epithelial and stromal cells in which lack of DNA synthesis prevailed when epithelial cells were cultured alone, but epithelial DNA synthesis occurred when these cells were co-cultured with stromal cells and this response was further enhanced by E2 or IGF1 (Pierro et al., 2001). However, these results to our knowledge have never been repeated, and indeed, effects were small and only shown for the incorporation of ^{3}H-thymidine and not on DNA synthesis into nuclear DNA per cell by autoradiography. Thus, the changes could be accounted for by effects on alterations in thymidine pools giving changed ^{3}H-thymidine specific activity. Nevertheless, at

face value the observations give some support for the importance of epithelial-stromal interactions in human endometrial growth and physiology similar to the mouse uterus.

Kurita et al. applied the tissue recombinant technique with reconstructed chimeric endometrium composed of human epithelial cells and mouse stroma from wild-type or ER null mice as well as solely human endometrium composed of human epithelial and stromal cells (Kurita et al., 2005). Both mouse/human and human/human tissue recombinants reorganized into endometrial-like tissue under the kidney capsule of the nude host mice and were functionally responsive to E2 (Kurita et al., 2005). Notably, the proliferative response of the endometrial epithelium to E2 differed between mouse and human tissue. While the mouse uterine epithelium reached maximum proliferative response to E2 already 16–18 h after administration, human uterine epithelium required 5–7 days to show maximal proliferation (Kurita et al., 2005). Interestingly, an extended 7 days of E2 treatment resulted in the inhibition of mouse epithelial cell proliferation in the presence of wild-type mouse stroma. Moreover, E2 was ineffective in inducing epithelial proliferation and increase in the expression of PGR when wild type mouse epithelium was reconstructed with stromal cells from mice lacking the ESR1 activation function-1 (AF-1) domain. In contrast, when mouse stromal cells from the ESR1 AF1 null mice were recombined with human epithelial cell, the epithelium responded with mitotic activity and increase in PGR (Kurita et al., 2005). These results suggest that there are species differences in uterine epithelial response to E2 and requirement for the estrogen receptor. Although hormonal responses may be similar, they are unlikely to be identical in the human and mouse uterus given the mouse estrous cycle lasts 3–4 days and the human menstrual cycle approximately 28 days. This observation confirms that a rodent model has limitations to further our understanding of endometrial biology in human.

3. Xenotransplant models of human endometrium

To underpin the molecular mechanism underlying endometrial biology, xenotransplants of human endometrial tissue to host mice have been developed. In one experimental model investigators have placed fragments of fresh endometrial tissue (1–2 mm^3 in size) in the subcutaneous space of host mice (here referred to as endometrial xenotransplants) (Alvarez Gonzalez et al., 2009; Coudyzer et al., 2013, 2015; Zaino et al., 1985; Bergqvist et al., 1985) or allowed fragments of the human endometrium to adhere to an intact peritoneal surface (*meso*thelial layer) to form proliferating endometriosis-like implants (Nisolle et al., 2000a, 2000b). In the other endometrial xenograft model, endometrial epithelial and stromal cells are enzymatically dissociated and then these cells are recombined in a rat-tail collagen gel and placed under the vascular kidney capsule of host mice to reconstruct the human endometrium (Fig. 1: here referred to as endometrial xenografts) (Masuda et al., 2007; Polotsky et al., 2009). The capacity of endometrial tissue to transplant appears not to depend on the timing when the fresh endometrium was harvested (Nisolle et al., 2000a); however most researchers sample the human endometrium during proliferative phase. The discovery of immunodeficient mice tremendously contributed to xenotransplantation research because these animals possess limited capacity to reject foreign tissue and thus are ideal hosts. Initial xenograft and xenotransplant models of the human endometrium used nude mice that have a defective thymus with T cell deficiency (Zaino et al., 1985; Bergqvist et al., 1985; Polotsky et al., 2009; Zamah et al., 1984). However, subsequently it was discovered that severe combined immunodeficiency (SCID) mice that lack functional T and B lymphocytes (Alvarez Gonzalez et al., 2009; Bosma et al., 1983) and, in particular, non-obese diabetic (NOD) /SCID/γc^{null} mice (NOG) (Ito et al., 2002) that additionally have defective NK cell activity and cytokine production showed higher engraftment rate and improved survival of grafted tissue (Masuda et al., 2007; Matsuura-Sawada et al., 2005). RAG-2/γ(c) mice that lack T and B lymphocytes as well as NK cells, but can produce cytokines were shown to be more suitable for experiments of long duration because they tend to avoid early graft rejection and therefore have prolonged survival of the grafted tissue. Indeed, Greenberg and Slayden reported maintenance of the endometrium-like morphology in subcutaneous xenotransplants up to the length of 4 hormonally induced menstrual cycles (Greenberg and Slayden, 2004).

As mentioned earlier there are various types of *in vivo* research models of the human endometrium. Both xenotransplant and xenograft models of the human endometrium vary based on strains of mouse used, and whether fresh or digested endometrial fragments are used. Morphological difference at the histological tissue level has been noted among different host mouse strains. For instance tissue fragments of the human endometrium grown intraperitoneally or subcutaneously in the nude mice show various degree of fibrosis around the transplanted tissue (Bergqvist et al., 1985; Grummer et al., 2001); however such morphological features are not found in the endometrial xenotransplants and xenografts grown in the severely immunodeficient, SCID mice (Alvarez Gonzalez et al., 2009; Nisolle et al., 2000b; Masuda et al., 2007). Fibrosis around the grafted tissue may be detrimental for proper endometrial function by inhibiting adequate vascularization of the implanted tissue and therefore SCID mice appear to be a better host of both human endometrial xenotransplants and xenografts. However, it should be noted that we did not find significant fibrosis around our human endometrial xenografts when dissociated epithelial and stromal cells were recombined and placed under the kidney capsule of nude mice (Polotsky et al., 2009), thus suggesting variations in technique or graft site may play a role in this process.

Engraftment of the human endometrial biopsy fragments placed into the subcutaneous space or in intraperitoneal locations of host mice occurs already a few days after implantation. These xenotransplants survive for up to 4 weeks. While implantation and preservation of normal morphology of transplanted endometrial biopsy fragments is not dependent on sex steroid hormone, E2 is required for epithelial proliferation and expression of estrogen and progesterone receptors in the xenotransplanted tissue (Matsuura-Sawada et al., 2005; Grummer et al., 2001; Aoki et al., 1994). In contrast, when dissociated epithelial and stromal cells are recombined in rat collagen and placed beneath the kidney capsule of ovariectomized host mice, E2 is essential for engrafting as well as for adequate growth and differentiation of grafted endometrial tissue (Masuda et al., 2007; Polotsky et al., 2009). In this model endometrial-like tissue with well-delineated glands and stroma is found in the xenografted outgrowths typically after 3 weeks of estrogen treatment (Masuda et al., 2007; Polotsky et al., 2009) that is most effectively administered through subcutaneous hormone releasing pellets or oil deposits (Fig. 1). Matsuda et al. reported a 100% take rate of reconstructed xenografts of human dissociated endometrial epithelial and stromal cells in E2-treated NOG mice (Masuda et al., 2007) compared to 30–40% take rate reported in nude mice (Polotsky et al., 2009). The high take rate of xenografts in NOG mice was attributed to the profound state of immune deficiency of these animals. The advantages of the high take rates in NOG mice enable the use of one endometrial biopsy for several grafts, thereby enhancing the experimental homogeneity and efficiency. The timing of ovariectomy for the removal of endogenous sex steroid influence has varied between different studies. However similar take rates were reported regardless of whether the host animals were ovariectomized 1–2 weeks prior to, at the time of, or after the establishment of xenotransplants using endometrial tissue fragments.

4. Human origin of the endometrial xenotransplants and xenografts

Researches have used human specific markers to confirm the origin of xenotransplanted and xenografted endometrial tissue. For example, immunohistochemical staining of intraperitoneally implanted frag-

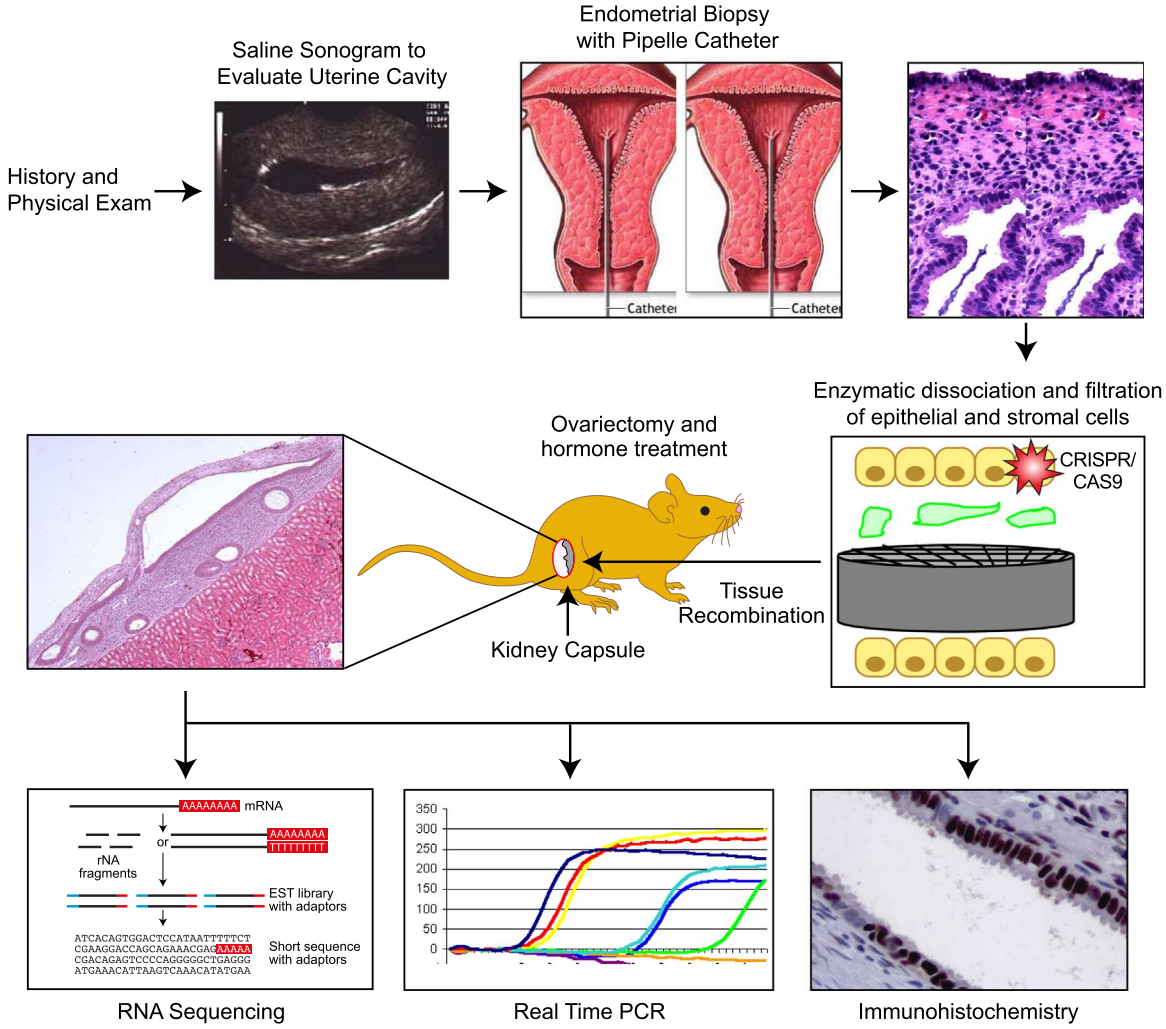

Fig. 1. Schematic of Human Endometrial Biopsy and Analysis. After screening and informed consent an endometrial biopsy is taken with a pipelle catheter from women volunteers usually in the proliferative stage of the menstrual cycle. These biopsies are washed in McCoy's media and minced into pieces and subjected to enzymatic dissociation with collagenase and DNase I at 37 °C with shaking as described (Polotsky et al., 2009). After digestion cells were collected by centrifugation and filtered through sterilized 85uM nylon mesh filters. Retained tissue from the first filtration was digested with 0.5% w/v hyaluronidase, 0.5% w/v protease and DNase I in McCoy's medium for 20 min. In this fashion, the epithelial cells are segregated as organoids containing several glands in an effort to preserve physiological tissue orientation (Kurita et al., 2005). The filtrate from the first filtration containing the stromal cells is then separated from the remaining red blood cells with a one-layer Percoll density gradient. In order to obtain a single cell preparation of stromal cells, the suspension was then passed through a sterilized 20 mm nylon mesh filter. In general, one endometrial biopsy yielded 10–20 million of epithelial and stromal cells sufficient for grafting 10–20 hosts. This method also yields pure isolates of stromal and epithelial compartments of human endometrium for analysis. In principle these cells can then be transfected or infected with lentiviruses to allow genetic manipulation using for example, the CRISPR/CAS9 system. However, to date this has only been performed to enable luciferase expression. To prepare xenotransplants freshly isolated uterine stromal cells and epithelium are recombined in 30 mL of rat-tail collagen gel by placing three to five epithelial organoids and approximately 500,000 stromal cells per tissue recombinant. The recombinants are allowed to gel under room temperature. Kidney grafting into nude mice is conducted *via* a dorsal incision under anesthesia (http://mammary.nih.gov/tools/mousework/Cunha001/index.html). One xenograft is implanted under each kidney capsule of each adult intact 8–10-week-old female nude mice. Usually mice are ovariectomized at the same time and an implant of E2 is given. After allowing 6 weeks for tissue outgrowth the implant is removed and the mice rested before hormonal treatment (Fig. 2). These procedures give a xenotransplant with recognizable endometrial structures containing epithelial glands and stroma overlaid on the kidney of the mice as shown. Following hormone treatment the tissue can be analyzed using many different techniques including IHC, laser capture micro-dissection of individual cellular compartments and real time PCR or RNA sequencing to determine transcriptional changes. The method will become even more valuable as technology improves to use multi-parametric analysis on a single cell basis in tissue sections.

ments of human endometrial biopsies indicated staining patterns of glandular epithelial and stromal cells of exclusively human origin using antibodies specific for human cytokeratin and vimentin, respectively (Grummer et al., 2001). In agreement, Matsuda and colleagues performed immunofluorescence staining of human endometrial xenografts with human anti-vimentin known to recognize only human vimentin and confirmed the human source of reconstructed endometrial-like tissue (Masuda et al., 2007). Our group, on the other hand, performed *in-situ*-hybridization staining with a probe specific to human Alu-sequence and showed selective positive hybridization in the endometrial epithelium and stroma of the xenograft and none in the adjacent mouse kidney parenchyma (Polotsky et al., 2009). Additionally, staining with Hoechst 33258 that shows distinct morphology for mouse and human nuclei can be used to confirm human origin of the endometrial grafts (Cunha and Vanderslice, 1984). These methods indicate that the human endometrial xenotransplants and xenografts have largely preserve the endometrial cellular composition of their original species source.

5. Neovascularization of human endometrial xenotransplants and xenografts

Angiogenesis is the process by which new microvessels develop from the existing vessels and is essential for the survival and growth of the human endometrial grafted tissue (Nap et al., 2004). The origins of neovasculature in endometrial xenotransplants xenografts remain

controversial and may in fact vary depending on the strain of the host mice (Alvarez Gonzalez et al., 2009; Masuda et al., 2007; Grummer et al., 2001; Bruner-Tran et al., 2002a; Eggermont et al., 2005). In the earlier studies, endometrial lesions transplanted into a nude or SCID mice were reported to obtain their vascular supply from the surrounding host vascular network, whereas the pre-existing vessels of human origin gradually disappeared (Grummer et al., 2001; Bruner-Tran et al., 2002b). Grummer et al. stained vessels of human origin with human specific anti-vWF antibody and mouse endothelia of a rat anti-mouse pan-endothelial cell antibody and demonstrated that the vessels within endometrial transplants were only of mouse origin (Grummer et al., 2001). In contrast, Masuda et al. demonstrated vessels of human origin throughout the endometrial xenografts that were reconstructed by recombining dissociated epithelial and stromal cells and placing them under the kidney capsule of NOG mice (Masuda et al., 2007). Moreover, these human-derived vessels were shown to invade into the mouse kidney parenchyma where they become connected to mouse vessels, thus forming chimeric vessels (Masuda et al., 2007). Because the NOG mice are severely immunodeficient and lack NK cells, it was proposed that the lack of NK cells allowed the neovascularization of vessels of human origin within the xenografts. However, Alvarez Gonzalez et al. subsequently showed vessels of human origin within the human xenotransplants implanted subcutaneously in both nude and SCID mice (these immunodeficient mice have intact NK cells). Additionally, throughout the endometrial xenotransplants they observed chimeric vessels in which vessels staining positive for human markers were connected to those of murine origin (Alvarez Gonzalez et al., 2009). It is plausible that the location of the ectopically reconstructed endometrium and adjacent vascular environment also influences how and from which source the neovascularization of the graft develops. However, in no cases have the mechanisms behind the vascularization of the grafts been analyzed.

6. Xenotransplanted human endometrium responds to sex steroid hormones

In order for xenotransplanted and xenografted endometrial tissue to undergo similar dynamic changes to that of human eutopic endometrium under the influence of sex steroids, their cognate steroid hormone receptors, ESR1 and PGR need to be expressed in the tissue. In ovariectomized and untreated host mice, immunohistochemical staining of endometrial xenotransplants with anti-ESR1 demonstrated robust expression of ESR1 in glandular epithelium and weaker staining in the stroma (Polotsky et al., 2009). It is well established that while E2 down-regulates its own receptor, ESR1, in the normal human endometrium, it increases PGR expression. Consistent with the human eutopic endometrium, treatment of ovariectomized host mice with E2 resulted in down-regulation of ESR1 in the endometrial xenografts of dispersed and recombined epithelial and stromal cells compared to those without hormonal exposure (Polotsky et al., 2009). The treatment with E2 also resulted in up-regulation of PGR in both epithelial and stromal compartment of these human endometrial xenografts (Masuda et al., 2007; Polotsky et al., 2009). Furthermore, glandular epithelial cells became pseudostratified and stained positive for anti-MCM2 and anti-MKI67 (markers of cell proliferation) as an indication of E2-induced cell proliferation (Masuda et al., 2007; Polotsky et al., 2009). The endometrial responses of the xenografts to E2 are thus comparable to those found in eutopic human endometrium.

Menstruation, that is cyclic shedding of the functionalis layer of the endometrium induced by P withdrawal, is a unique event of the human and Old World primates. Models of menstruation have successfully been developed based on xenotransplantation of human endometrial biopsy fragments into the subcutaneous space of SCID (Guo et al., 2011) and NOG host mice (Matsuura-Sawada et al., 2005) and in endometrial xenograft models reconstructed using dispersed epithelial and stromal cells that are recombined and implanted under the kidney capsule of NOG mice host mice (Masuda et al., 2007). After formation of xenotransplants and xenografts, ovariectomized host animals were cyclically treated with E2 for 2–3 weeks and then E2 plus P for another 14 days to induce an artificial menstrual cycle (Masuda et al., 2007; Matsuura-Sawada et al., 2005). In these models E2 administration induces proliferative changes in endometrial epithelial cells whereas E2 plus P treatment inhibit this cell proliferation and additionally transforms epithelial cells into the secretory phase. Furthermore, P treatment induced predecidualization changes in stromal compartment of E2-primed xenotransplants and reconstructed xenografts (Masuda et al., 2007; Matsuura-Sawada et al., 2005). In particular, stromal edema and increased expression of prolactin, a marker of decidualization, were observed in stromal cells that morphologically had transformed to typical large and round decidualized cells (Masuda et al., 2007). Similar to human decidualized endometrium a large number of CD45-positive leukocytes, including CD56-positive NK cells and CD14-positive cells, both of human origin, has been reported to be abundant in the grafted tissue following 28 days of hormone treatment in NOG host mice (Masuda et al., 2007).

In ovariectomized mice, destruction of xenografted stromal tissue and glandular structures as well as sloughing of luminal epithelium can be seen approximately one week after withdrawal of P in the human endometrial xenografts that were created by recombining dispersed epithelial and stromal cells and which were implanted under the kidney capsule of the NOG host mice (Masuda et al., 2007). Also, blood-filled cysts are formed in these endometrial tissue reconstructs because the lumen of the endometrial xenotransplant lacks an outflow tract preventing the escape of blood (Masuda et al., 2007). Interestingly, after the phase of “menstrual” destruction following the cessation of E2 and P4 supplementation, endometrial xenotransplants created using endometrial fragments into the subcutaneous space of ovariectomized NOG host mice quickly return to proliferative phase (Matsuura-Sawada et al., 2005). These results not only suggest that the early growth phase of the endometrium is indeed independent of estrogen, but also indicates that xenotransplants of the human endometrium have significant regenerative capacity similar to the eutopic endometrium. Traditionally, the human endometrium is believed to regenerate from the basalis layer that persists after menstruation (Padykula, 1991). However, the basalis layer is absent from xenotransplants and yet these lesions have significant regenerative capacity, which raises the possibility that endometrial stem/progenitor cells present in the functionalis layer may contribute to the physiological regeneration of the endometrium. Further research is needed to clarify this possibility.

7. Functional studies using xenotransplanted human endometrium

The endometrial xenotransplant model using dissociated epithelial and stromal cells that are recombined beneath the kidney capsule of immunodeficient mice presents a novel experimental opportunity to investigate endometrial functions. For example, Matsuda et al. lentivirally engineered human dispersed endometrial cells to express luciferase prior to grafting and in this fashion non-invasively and quantitatively were able to track hormone induced cellular changes by *in vivo* bioluminescent imaging (Masuda et al., 2007).

In our previous studies, we used reconstructed xenograft model of dissociated human endometrial epithelial and stromal cells into the kidney capsule of nude mice to define molecular mechanisms underlying endometrial epithelial cell proliferation by E2 (Polotsky et al., 2009). Specifically, we investigated whether similar cellular mechanisms were operative in the human endometrium as previously discovered in the mouse uterus. In the mouse, inhibition of glycogen synthase kinase 3ß (GSK3ß) under the influence of E2 is a key event that results in the nuclear localization of cell cycle regulatory molecule, cyclin D1 in the uterine epithelium (Chen et al., 2005). Nuclear cyclin D1, in turn, together with its partners CDK4 and CDK6 phosphorylates

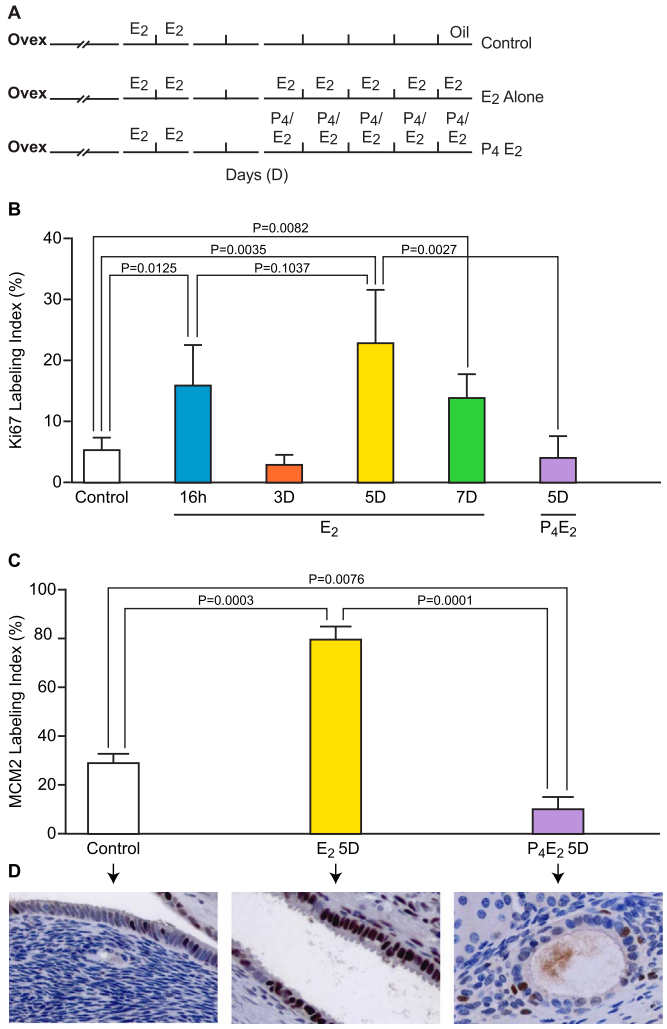

Fig. 2. In human tissue xenotransplants E2 induces two waves of DNA synthesis and this is inhibited by Progesterone. (A) 6 weeks after transplantation E2 implants are removed and mice rested for two weeks. Mice after priming with two daily subcutaneous injections (sc) of 100 ng E2/mouse were divided into groups of two to five animals per group according to the following sc treatments: 5 days of daily: (a) 0.1 mL peanut oil; b) 125 ng of E2 in peanut oil; c) 1 mg Progesterone (P4) plus 125 ng E2 in peanut oil. Animals were killed at the times indicated after the first E2 injection (except for E2 that was also given for 7 days). Each experiment was repeated at least twice. (B) After the hormonal treatments shown mice were killed, kidneys isolated and xenotransplants fixed. After embedding and sectioning at 5 μM they were then subjected to immunohistochemistry for MKI67 as described (Polotsky et al., 2009). The percent of nuclear positive cells in the epithelial compartment was determined according to hormonal treatment. Maximal E2-induced DNA synthesis was at 5 days and this was inhibited to control levels by concurrent treatment with P4. Statistical analysis shown by ANOVA. (C) The same sections as in B were also immunostained for MCM2 as described (Polotsky et al., 2009). MCM2 nuclear positive cells shown below in the representative histology (D) were enumerated for control oil treated mice, E2 treated mice for 5 days and P4E2 mice for 5 days. Positive immunohistochemical nuclear stain given by anti-MCM2 antibody is brown in 5 μM sections counter stained with Haematoxylin for the treatments indicated by the arrows. E2 significantly increased the number of MCM2 positive cells to ~80% and this is inhibited to below the control level by P4 treatment. Students T test $P > 0.05$ considered significant.

pRB with subsequent dramatic increase in cyclin A and elevation of CDK2 causing further phosphorylation of pRB, coincident with progression of epithelial cells towards S-phase of the cell cycle, thus DNA synthesis (Chen et al., 2005). In the mouse uterus, our group previously showed that targeted inhibition of GSK3ß by a chemical inhibitor, lithium chloride was permissive for uterine epithelial cells to undergo DNA synthesis even in the absence of E2 (Chen et al., 2005). In this context, we biochemically inhibited GSK3ß by adding lithium to the drinking water of the host nude mice carrying the endometrial xenografts (Polotsky et al., 2009) which resulted in nuclear accumulation of cyclin D1 and proliferation of epithelial cells of the xenografts in the absence of E2 (Polotsky et al., 2009). These results indicate that similar to the mouse uterine epithelium E2 signaling occurred through GSK-3ß inhibition in the epithelium of the human reconstructed endometrial xenograft.

Using an identical xenograft model in nude mice, we have further explored the proliferative response of the human endometrial epithelium to E2 and P. In these experiments, the host nude mice were treated with E2 or E2 plus P in regimens designed to mimic the human menstrual cycle (Fig. 2A), followed by immunohistochemical staining of the xenografs with MKI67 antibody (marker for cell proliferation) at different time points (Fig. 2B). In the E2 treated group compared to the untreated group, the first proliferative response in the epithelial cells was observed after 16 h of treatment. Despite the continued E2 treatment, after 3 days of E2 treatment, the epithelial cells reduce their proliferation and the proportion of the epithelial cells proliferating returned to that of untreated control epithelial cells. Interestingly, a second and maximal wave of proliferation occurred after 5 days of E2 with approximately 22% of epithelial cells staining positive to MKI67 compared to the control epithelial cells. P reduced this proliferative response to untreated control levels when E2 and P were co-administered to the animals for five days (Fig. 2B). This indication of two waves of DNA synthesis in response to E2 in human endometrial epithelium (Fig. 2B) is reminiscent, although on an expanded time-scale to that seen in mice (Martin et al., 1973). Detection of such kinetics would be impossible to detect in human endometrial biopsies, but this observation aligns responses in mouse and humans together.

Analysis of mini-chromosome maintenance protein (MCM)2 expression that is a pre-replication licensing factor essential for DNA synthesis and whose concentration, nuclear localization and activity is stimulated by E2 but inhibited by P, also showed a similar readout but with a greater percentage of cells staining positive for nuclear MCM2 than MIK67 (Fig. 2C,D). Thus, MCM2 may represent a more sensitive indicator of DNA synthesis than MKI67. It also shows again a similarity with the stimulation of mouse uterine DNA synthesis by E2 and inhibition by P. Indeed the inhibition of E2 induced DNA synthesis by P shows that these xenotransplants are not P insensitive as observed in endometriosis (Kao et al., 2002). Thus, they appear to more closely represent functional endometrium than transplants of endometrial tissue into the peritoneal cavity that resemble implants of endometriosis. Interestingly, in mice the regulation of MCM2 expression is in part controlled by the Kruppel-like transcriptions factors (KLF) 4 and 15 with the latter mediating the negative inhibition by P (Ray and Pollard, 2012). In human endometria KLF15 has recently been shown to be deregulated in eutopic endometria of patients with endometriosis (Kim et al., 2015). These patients are P insensitive suggesting that similar mechanisms to those observed in mice are present in humans. Manipulation of these human endometrial xenografts could allow this observation to be examined experimentally and perhaps also to indicate therapeutic opportunities in humans.

The xenografted stromal compartment regenerates to surround endometrial glands; however, in our series using nude mice as a host, the abundance of the stroma remains limited and therefore this model appears to be better suited for the study of the luminal and glandular epithelium. Indeed we have had difficulty in showing reliable stromal proliferative responses to E2 and P. Despite the extensive regenerative capacity of dissociated and recombined endometrial epithelial and stromal cells, the main disadvantage of the human endometrial xenograft model is the limited amount of tissue that can be harvested from each tissue outgrowth. For this reason, the genetic material needs to be amplified prior to gene expression profiling; and moreover, quantitative protein assays including Western blot and large-scale proteomics are not to date feasible (Fig. 1).

8. Xenotransplants and xenografts of the human endometrium for testing of compounds

The idea that the effect of novel compounds on the human endometrium could be tested using an *in vivo* model without giving them to women is quite intriguing. In our experiments as described above, we administered lithium chloride to host animals to inhibit GSK-3ß and investigated its effect on endometrial cell proliferation. Even though lithium is a therapeutic agent used for bipolar disorder, it has potential renal toxicity and accordingly, its administration to volunteer women solely for research purposes would be unethical. Amaya et al. studied the effect of resveratrol (a natural phytoestrogen found in red wine, grapes, and berries) on endometrial cell proliferation in the xenotransplant model of endometrial fragments placed in the subcutaneous space of the host RAG-2/γ(c) mice (Amaya et al., 2014). Compared to the E2 treated group, E2 plus a high dose resveratrol treatment decreased immunostaining of MKI67 and ESR1 in the epithelium of the xenotransplants, suggesting this compound reduces epithelial proliferation possibly through downregulation of ESR1, and therefore acts as a partial estrogen antagonist in the endometrial epithelium (Amaya et al., 2014). In another study the investigators examined the endometrial effects of the progestogen, levonorgestrel (LNG) that is a highly efficient contraceptive in the form of a LNG releasing intrauterine system (IUS). In this study the authors used fragments of human endometrium that were implanted in subcutaneous space of SCID host mice to create endometrial xenotransplants (Alvarez Gonzalez et al., 2009). Subsequently, mice carrying these endometrial xenotransplants were treated with E2 or E2 plus LNG for a total of 4 weeks after which endometrial morphology was evaluated and compared to human eutopic proliferative endometrium and human endometrium exposed to LNG-IUS. After 4 weeks of treatment, all xenotransplants from the mice treated with LNG presented morphology and cell proliferation pattern similar to those observed in the endometrium exposed to LNG-IUS. These features included decreased glandular epithelial cell proliferation, atrophy of glandular cells and stromal decidualization (Alvarez Gonzalez et al., 2009). Similarly, the steroid receptor status of the xenotransplants in LNG-treated mice mimicked the expression pattern to the LNG-IUS-treated human eutopic endometrium, including decrease in stromal and glandular PGR expression and reduction in stromal ESR1 (Alvarez Gonzalez et al., 2009). Hence, there are several examples describing the feasibility of human endometrial xenografts and xenotransplants as an invaluable tool to study endometrial effects of various compounds. These models are particularly advantageous when there are practical and ethical limitations in administering compounds to humans. Moreover, this *in vivo* animal model can be suitable to evaluate possible efficacy of various therapeutic modalities for endometrial disease prior to testing in women with these conditions.

9. Concluding remarks

Decades of research has revealed a critical role of paracrine signaling in regulating endometrial cell growth and differentiation that has led to the development of *in vivo* models for the study of the physiology of normal endometrium. When recombined, isolated human epithelial and stromal cells have the capacity to regenerate and reconstitute the endometrium ectopically with normal tissue organization, endometrial cellular components and responsiveness to sex steroids. The capacity of endometrial xenografts to respond to steroid hormones and regenerate confirms this model as a powerful tool for the study of hormone effects in endometrial tissue over the course of a menstrual cycle. A real benefit of the xenotransplants and xenograft models is that one endometrial biopsy provides tissue for several endometrial xenografts, thus limiting the number of women needed for biopsy, yet allowing a decent number of experimental replicates. Tissue xenograft of the human endometrium offers means to define molecular factors and pathways operational in endometrial physiology, including control of cell proliferation, stromal decidualization and degeneration. Importantly, the model provides novel avenues for the study of human endometrial biology that were not previously possible because the xenograft model using dispersed endometrial cells recombined under the kidney capsule of recipient mice allows application of manipulative molecular tools, such as CRIPS-CAS9 technology to knock-out genes of interest in cell-specific fashion in xenografted tissue (Fig. 1). In addition, the dramatic advances in genetic sequencing technology and multi-parameter protein detection at the single cell level will allow the state of individual cells to be interrogated *in vivo* in a controlled hormonal environment. Furthermore, advances in organiod culture technology of endometrial glandular epithelial cells and endometrial fibroblasts raises the possibility of genetic manipulation, selection for positive alterations, followed by recombination into functional hormonally responsive xenografts into the kidney capsule followed by endocrine treatment to define regulated pathways controlling proliferation and other endometrial functions. These new exciting techniques will surely lead to rapid advances in our understanding of human endometrial biology. Accordingly, these investigational tools holds promise to yield mechanistic discoveries of endometrial functions in human that will be critical for understanding the etiology of endometrial pathologies and ultimately the development of targeted therapies of endometrial disease. Moreover, this model is proposed for the assessment of potentially harmful biochemical compounds, such as novel drugs, that may impact the endometrium, but cannot be administered to women.

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